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REVIEW

Biological hydrogen production: molecular and electrolytic perspectives

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Abstract

Exploration of renewable energy sources is an imperative task in order to replace fossil fuels and to diminish atmospheric pollution. Hydrogen is considered one of the most promising fuels for the future and implores further investigation to find eco-friendly ways toward viable production. Expansive processes like electrolysis and fossil fuels are currently being used to produce hydrogen. Biological hydrogen production (BHP) displays recyclable and economical traits, and is thus imperative for hydrogen economy. Three basic modes of BHP were investigated, including bio photolysis, photo fermentation and dark fermentation. Photosynthetic microorganisms could readily serve as powerhouses to successively produce this type of energy. Cyanobacteria, blue green algae (bio photolysis) and some purple non-sulfur bacteria (Photo fermentation) utilize solar energy and produce hydrogen during their metabolic processes. Ionic species, including hydrogen (H^+) and electrons (e^-) are combined into hydrogen gas (H_2), with the use of special enzymes called hydrogenases in the case of bio photolysis, and nitrogenases catalyze the formation of hydrogen in the case of photo fermentation. Nevertheless, oxygen sensitivity of these enzymes is a drawback for bio photolysis and photo fermentation, whereas, the amount of hydrogen per unit substrate produced appears insufficient for dark fermentation. This review focuses on innovative advances in the bioprocess research, genetic engineering and bioprocess technologies such as microbial fuel cell technology, in developing bio hydrogen production.

Keywords Photo fermentation · Bio photolysis · Dark fermentation · Microbial electrolytic cells

Introduction

Fossil fuels including oil, natural gas and coal maintain a finite lifetime. Over the years it has been observed that these energy sources have also caused, and are still causing, severe damage to the earth's atmosphere (Abdalla et al. 2018). Although they substantially contribute to an easy life style, fossil fuel limitations also prevail among the existing and future generations. Indirect contribution of these fossil fuels towards inequality in society is evident by the fact that over 50% of its consumption is by wealthier populations compared to those of lower socio-economic communities. Nevertheless, abruptly ending the use of these fossil fuels is controversial and could potentially create an energy crisis (Head and Gray 2016). Exploration of alternative energy sources, and accumulation of vast power supplies are promising approaches toward solving this crisis. Hydrogen is a valuable gas as a clean energy source and as feed stock for some industries. It is a high energy (122 kJ g^{-1}) clean fuel, non-pollutant gas in the environment; hence hydrogen can be used for many different purposes. Based on these advantages, vehicle manufacturers have demonstrated that hydrogen can be used directly in an internal combustion engine or used as a fuel cell in the automobile industry. Therefore demand on hydrogen production has been increased considerably in recent years (Lecker et al. 2017). Hydrogen fulfills the following given facts;

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(i) On earth, hydrogen is the most abundant element as a component of water and various other organic compounds.
(ii) When combusted with molecular oxygen the reaction is exergonic and produces heat—1 g of hydrogen produces 30 kcal of energy compared to gasoline (11 kcal).
(iii) Combustion causes no atmospheric pollution, as the byproduct is mostly water (or the oxides of nitrogen in case of internal combustion engine), therefore eco-friendly.

which makes it a highly desirable preference for being considered as an alternative fuel source.

The vision of using hydrogen as a carbon neutral energy source is denoted by the term "hydrogen economy" (Bičáková and Straka 2012; da Silva Veras et al. 2017; Nikolaidis and Poullikkas 2017; Singh and Wahid 2015; Wang et al. 2017). It refers to replacing fossil fuels as a means for transportation and also as heating fuels. This involves three important objectives;

(i) Production and distribution;
(ii) Storage and transport;
(iii) Utilization in the form of fuel and/or as electricity.

Aspects of these objectives are discussed in detail below, in order to understand new inventions in the process of achieving increased hydrogen production.

Hydrogen production strategies

Chemical processes including electrolysis, steam reforming of hydrocarbons and auto thermal process are the most commonly used processes for hydrogen production. Fossil fuels such as natural gas, coal and liquid hydrocarbons are frequently used to produce hydrocarbons, which constitutes around 95% of hydrogen production. Remaining 4% represents the electrolysis of water and approximately 1% represents the biologically produced hydrogen (Fig. 1a). Moreover, chemical reforming of hydrocarbons and electrolysis of water are highly expensive techniques and replacement by cost effective methods is essential. In this regard, BHP using bacteria is a highly regarded method, which renders economical production of hydrogen. BHP stands out as an environmentally sound process carried out under mild operating conditions, using renewable resources (Wang et al. 2017; Wei et al. 2018). Several types of microorganisms such as the photosynthetic bacteria, cyanobacteria, algae or fermentative bacteria are commonly utilized for BHP (Fig. 2). Chemo heterotrophic species such as *Clostridium* and *Enterobacter* undergo dark fermentation, a light-independent process. This involves anaerobic fermentation of carbohydrates or other organic substrates, which results in the formation of hydrogen by the capture of electrons (generated upon the substrate catabolism), by protons. Likewise, a light dependent fermentative process called photo fermentation is also shown to produce hydrogen, with the involvement of non-oxygenic photosynthetic bacteria, such as purple

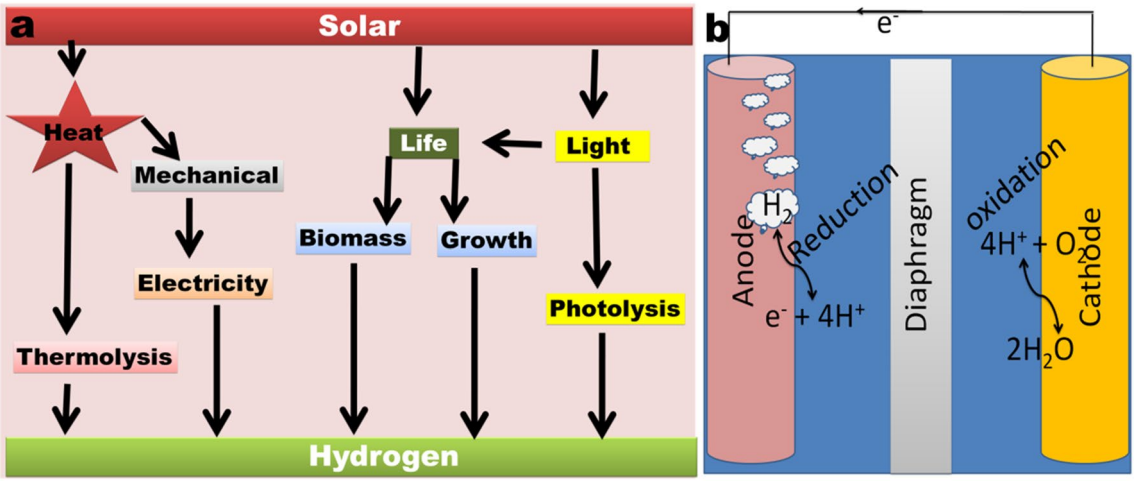

Fig. 1 **a** Various modes of hydrogen production are shown. Thermolysis and electrolysis of water is done by utilizing the heat and electric energy. Biological production of hydrogen represents the more sustainable mode, which is powered by the free energy derived from the sun. Solar energy, predominantly in the form of light energy is harvested by photo-autotrophs in the form of biomass. Some of these organisms (exclusively micro-organisms) generate hydrogen as a part of their metabolism. Photoelectrolysis of water is also done to harvest hydrogen, as shown in the panel **b** in the figure

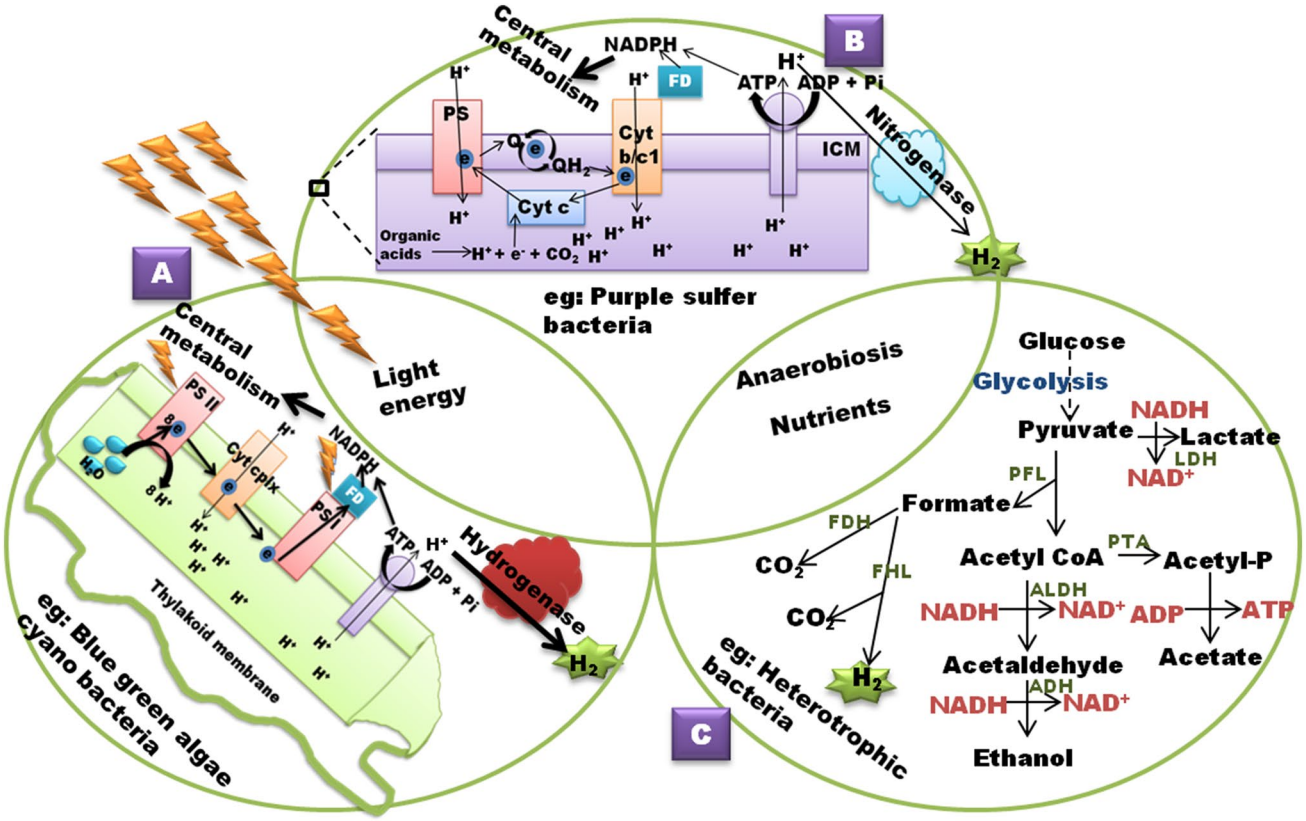

Fig. 2 Shows the mechanism of biological hydrogen production. In the case of bio photolysis (**a**), light energy is converted in to chemical energy in the thylakoid machinery present in the membrane of phototropic organisms such as *cyanobacteria* and blue green algae. **b** Purple sulfur bacteria mainly undergo photofermentation with light harvesting complexes present in photosystems. Protons released in both these processes are converted to hydrogen by means of hydrogenases (represented in red) or nitrogenases (blue), respectively. **c** Heterotrophic microorganisms such as *E. coli* and *Clostridium* undergo dark fermentation, in anaerobic conditions hydrogen is evolved as a side product along with secondary metabolites that are formed by means of fermentation processes

non-sulfur and green sulfur bacteria. Photo fermentation involves the use of alternative reduced compounds, such as organic acids and hydrogen sulfide as electron donors, unlike carbohydrates as mentioned previously. Bio photolysis, a third mode of bio hydrogen production, is unique to photo autotrophic organisms, with green algae and cyanobacteria exhibiting the greatest propensity for hydrogen production (Fig. 2).

Processes such as bio photolysis, photo fermentation and dark fermentation constitute BHP methods and are vastly investigated for cost reductions. Previous reports, explicitly employing dark fermentation, photo fermentation, and bio photolysis to create BHP, suggest that axenic cultures of the oxygenic phototrophic bacteria *Synechococcus* sp. OU 103 and *S. cedrorum* are capable of producing hydrogen, and high yields were observed, using malate for the former species, and sulfide with the latter species, as electron donors (Sasikala and Ramana 1994). *Rhodobacter* sp. was also extensively studied for their ability to produce photo-biological hydrogen exhibiting potential for high hydrogen yields (Sasikala et al. 1990, 1992).

Strategies for improving hydrogen yields in 3 types of BHP systems

Systems producing hydrogen, namely through, bio photolysis, photo fermentation and dark fermentation, unfortunately fall far short of what is necessary to develop a practical system. There are several barriers to achieving substantial system improvement, for example in the first two processes (bio photolysis and photo fermentation); systems capable of converting captured solar energy into hydrogen require standardization. In the latter process (dark fermentation), the drawback is hydrogen yield per unit substrate being low (Table 1). Nevertheless, there are a number of strategies that are guiding current efforts to overcome these barriers (Hassan et al. 2018). In order to achieve high BHP, various parameters involved in all the three aforementioned systems

Table 1 Showing assessment of different biohydrogen processes

Process	Advantages	Disadvantages	Future scenario	References
Bio photolysis	1. H_2 produced directly from sunlight and splitting of water. Solar conversion energy increased 10 times as compared to trees, crops, etc 2. Nutrient provisions are low 3. H_2 is produced from water 4. Can fix N_2 from the atmosphere	1. Efficient utilization of light 2. Directing electron flux to H_2 production 3. Oxygen sensitivity of hydrogenases 4. Slow rate of H_2 production	1. Inter disciplinary research combining biology and engineering 2. Metabolic engineering and synthetic biology approaches for manipulation/optimization of reductant flux to hydrogen 3. Researching about existing compartments for H_2 production	Volgusheva et al. (2017), Kosourov et al. (2018) and Nagy et al. (2018)
Photo fermentation	1. Wide spectrum of light energy can be used 2. Choice of inoculums—wide range of organic-waste effluents can be used 3. Both hydrogenase and nitrogenase-basedH_2 production is present	1. High possibility of contamination by other H_2 utilizing organisms 2. Clear exploitation of light/dark cycles 3. Lagging knowledge about light availability/reactor design	1. Creating antenna mutants for efficient photo systems 2. Fundamental understanding of light/dark cycles 3. Metabolic modeling 4. Reactor design	Stephen et al. (2017), Krujatz et al. (2015) and Adessi et al. (2016)
Dark fermentation	1. Wide range of organic-waste effluents can be used as carbon substrates 2. Constant H_2 production could be achieved without light illumination 3. Precious secondary metabolites like butyric, lactic, acetic acids etc. could be produced as by products 4. O_2 limiting step is not there as it is anaerobic	1. Understanding and development of genetic systems 2. Lower stoichiometric yields of H_2 3. Available reactor design, better understanding of biomass convertibility 4. Presence of CO_2 in the gas mix	1. Metabolic engineering of new strains 2. Molecular engineering of hydrogenase gene 3. Designing modified bioreactors for microbial consortia	Sundara Sekar et al. (2017), Wong et al. (2018) and Mishra et al. (2019)

were exclusively explored and discussed in the subsequent sections of this review. Increasing quality of light absorption, protecting hydrogenase enzymes, and substrate limitation are the predominant methodologies to improve bio photolysis. Improved BHP in photo fermentation include reactor design, addition of heterocyclic compounds and changes in pH or salt concentrations. Metabolic engineering is the most predominant approach to augmenthead dark fermentative BHP. Moreover, considering the drawbacks of those three systems individually, integrative approaches are ideal towards overcoming such limitations as integration has maximum conversion efficiencies in the range of 12 mol of H_2/mol-glucose from the substrate. It is found that organic-rich biomass has great potential as a substrate for dark, photo and integrated dark-photo fermentative (IDPF) hydrogen production. Limited hydrogen yield in dark fermentation due to incomplete oxidation of organic acids can be overcome with IDPF (Hitit et al. 2017). IDPF can be accomplished in two possible modes i. Two stage system, where two reactors with different conditions are successively used to perform dark fermentation and photo-fermentation. ii. Co-fermentation system; where same reactor is employed to simultaneously perform dark-fermentation and photo-fermentation (Uyar et al. 2015). Co-fermentation systems are thought to be more cost-effective and easier to adapt to environmental fluctuation, among both modes (Pachapur et al. 2015).

Biophotolysis and photofermentation

A large compilation of rates for hydrogen production of both bio photolysis and photo fermentation have been published in terms of milliliters of hydrogen per liter of reactor (Baykara 2018). About 100-fold variation from 1 mL L^{-1} h^{-1} to more than 100 mL L^{-1} h^{-1}variation in the production rates were observed, where, bio photolytic systems were appreciably lower than those of photo fermentation systems. Moreover, photo fermentation stands out to be an effective cumulative hydrogen yield generator, even when compared to other BHP systems like dark fermentation (Zhang et al. 2019). Light utilization, at full solar power, is inefficient due to a number of factors. At high light intensities, light utilization efficiency by an individual cell is compromised partly because of photosynthetic antenna sizes that are optimized for sub-maximal light intensity. In addition, factors like limiting effective light penetration into cultures and self-shading impedes overall photosynthetic efficiencies by lesser or equal to 1% and often closer to 0.1% (Stephen et al. 2017). Increasing the total captured spectrum and the quantity of light along with productive usage at high light intensities are the factors that seek improvement. Reduction of antenna sizes by creating antenna mutants, which are most efficient at highlight intensities, is a most probable initiative to avoid this problem (Polle et al. 2002; Singh and Das 2018).

Inhibition of oxygen evolution is one approach in order to protect the sensitive hydrogenases. One way to achieve a transition from stage 1 to stage 2 in photo-phosphorylation is reversible inactivation of photosystem II (PSII) and O_2 evolution. This was first reported upon sulfur deprivation of the green alga, *Chlamydomonas reinhardtii* (Melis 2007). Prolonged hydrogen production was observed in sulfur-deprived cells; conversely, this procedure is not deemed practical, as lack of oxygen inhibition is achieved only by 90% reduction in photosynthesis. Since the small amount of oxygen produced is consumed by respiration, the culture was growing anaerobically. Sulfur deprivation in the cyanobacterial species is done in two steps. The first is sulfur-replete stage and second sulfur depletion in the media to enhance anaerobiosis. In an interesting study by (Morsy 2011), two cyanobacterial species *C. reinhardtii* and *Spirulina platensis* were analyzed for studying the role of acetate from that of sulfur deprivation on creating anaerobiosis for hydrogen production. The result showed, in the case of *Spirulina platensis*, sulfur deprivation alone is sufficient for anaerobiosis and hydrogen production. Whereas, acetate alone installed anaerobiosis, followed by sulfur deprivation enhances the hydrogen production for the chlorophycean *C. reinhardtii*. Analogous techniques to sulfur deprivation are using depletion of other important nutrients. Deficiencies of elements such as magnesium, nitrogen, phosphorus and potassium have been examined to achieve similar or increased sustainability in BHP with *C. reinhardtii* (Volgusheva et al. 2015). During more recent analysis, comparison of BHP capacity and physiological status of the S and Mg- deprived cells was performed. Both deprivation protocols were carried out under similar experimental conditions, where different biochemical and biophysical measurements were imposed on the cells. The rates of photosynthesis, respiration, the accumulation of important photosynthetic and respiratory proteins, and the energy distribution between PSII and PSI were evaluated in both S- and Mg-deprived cells. Prolonged H_2 photo production was reported upon Mg-deprivation with 60% more H_2 (2.24 mmol g^{-1}) than S-deprived cells. This was attributed to the less drastic effects of Mg-depletion on metabolic performance of the cells and the elevated water splitting and O_2 uptake processes in the chloroplast (Volgusheva et al. 2017).

Nevertheless, looking in to the alternative hypothesis in hind sight, there are several other factors in combination with oxygen insensitivity that are responsible for cessation of hydrogen production. These include electron loss to either carbon fixation, oxygen reduction within the chloroplast or a cyclic electron flow (Godaux et al. 2015). Recent studies indicated that the electron loss is responsible for immediate hydrogenase inactivity, rather than oxygen exposure (Milrad et al. 2018). Hence, engineering a continuous process of hydrogen production can be achieved through bypassing

electron competition. In a concurrent study, higher efficiencies (0.9% Photo-synthetically active radiation—PAR) of photo-biological hydrogen was reported with nutrient deprived—alginate entrapped cells (Kosourov and Seibert 2009). A polymeric layer, being an alginate layer in this case, acted as a barrier to limit the oxygen diffusion in to the entrapped cells in the head space which has had potential for the scale-up as reported by the authors. A breakthrough protocol from the same group, for sustaining efficient H_2 photo production in *C. reinhardtii* was achieved by transferring growing cultures from continuous light to a train of strong light pulses superimposed on darkness or low background illumination. The authors reported an overall increase in the H_2 photo production yield, which is attributed to the redirection of the photosynthetic electron flow to hydrogenase, instead of CO_2 fixation and biomass formation (Kosourov et al. 2018). In another interesting study, short anaerobic induction was followed along with a substrate limitation to keep the Calvin–Benson–Bas—sham cycle inactive. In this alternative sulfur deprivation procedure, additional steps involved the application of a catalyst to remove the O_2 during water splitting. The authors reported higher amount of H_2 photo production compared to the sulfur-deprivation procedure, that supports continuous hydrogen production (Nagy et al. 2018). A sum of 19.4 mmol g^{-1} 1 (dry wt) of photo-biological hydrogen was observed when incubated under an argon atmosphere from non-heterocystous, filamentous cyanobacterium, *Lyngby aperelegans* in mid-exponential phase (Kaushik and Anjana 2011). This could be related to the accumulation of appreciable amounts of glycogen at this point in the culture. Further fourfold enhanced hydrogen production was reported from the same authors when the cells were immobilized in agar cubes or alginate beads compared to free culture (Anjana and Kaushik 2014). Taken together, electron competition path ways could be cleverly manipulated to direct the electrons towards hydrogenases. Metabolic engineering of the efficient cell factories that evolve H_2 can be another area to explore new vistas in BHP. Thus, re-directing the photosynthetic electrons to the targeted metabolic pathways and biofuel products, instead of biomass enhances BHP with photo fermentation.

Genetic engineering of molecular machinery involved in BHP is yet another exciting methodology being explored indicating promising results in H_2 yield. It was well established that D1 protein, a key component of photosystem II complex, was shown to be inhibited during the process of improved photobiological H_2 production. *Chlamydomonas reinhardtii* D1 mutant with reduced total chlorophyll was shown to produce more hydrogen with higher photosynthetic capacity compared to wild type (Scoma et al. 2012). More recently, heat-inducible artificial miRNA expression system targeting D1-encoded gene, psbA cassette was designed. This transgenic alga accumulated about 60% more H_2 content than the wild-type strain (Li et al. 2018). Codon optimized ferrochelatase (hemH) and leghemoglobin (lba) were expressed in the chloroplast of *Chlamydomonas* (strain cc849) to improve overall hydrogen production. The result showed that the mutant strain exhibited fourfold increased hydrogen production rates at low light intensities of 50 uEm^{-2} s^{-1} PAR, compared to the wild-type. This could be attributed to the presence of very low partial pressure of oxygen within the cells attributable to the stability and performance of the codon optimized proteins (Wu et al. 2011). Another problem for bio photolysis is the oxygen sensitivity of hydrogenases. Simultaneous evolution of oxygen and hydrogen requires a proton activating catalyst such as (FeFe) hydrogenases that can function at saturating, or even super saturating oxygen levels. FeFe hydrogenases are irreversibly destroyed by low concentrations of oxygen even during short time exposure. This represents the major fundamental obstacle to this approach, and efforts are underway to attempt to engineer hydrogenases that are less sensitive to oxygen (Hallenbeck et al. 2012; Nagarajan et al. 2017). With the addition of bulky side chains in the protein channel, passage of gasses like oxygen or hydrogen to the active site could be restricted. This was indicated by the molecular dynamics and X-ray crystallographic structures, that have shown these channels buried in the core of the protein. Moreover, these give plausible thought that mutations that narrow this channel could favor hydrogen diffusion over oxygen diffusion, leading to an improved protein (Stripp et al. 2009). Co-cultural studies give deep insights about formulating synthetic consortia of microorganisms, which is an interesting approach to achieve significant production of bio products. Co-culture of the mutant straincc849 along with *Bradyrhizobium japonicum* showed even more hydrogen production of 14-fold compared to the wild type cells alone (Wu et al. 2012). This increase in yield is attributed to oxygen scavenging ability of *Bradyrhizobium japonicum*, which forms root nodules and have abundant lba's that have high affinity for O_2 and carry that to fulfill respiration metabolism. More recently, co-culture of *C. reinhardtii* and *Thiomonas intermedia* was systematically investigated for enhancing the photobiological hydrogen production. With the application of sulfites, this co-culture showed more sustained and 5.9 times higher H_2 production (255.52 µmol mg^{-1} Chl) than that of a pure algal culture (Ge et al. 2019).

Purple non-sulfur (PNS) photosynthetic bacteria only present a single photo-system and are incapable of performing water splitting photosynthesis. This characteristic, along with their capacity to capture solar energy to execute the conversion of substrates to hydrogen with an additional energy input (e.g. acetate or lactate), has led to considerable studies of their use in BHP. Additionally, their function as part of two-stage systems for deriving additional hydrogen from the effluents of dark, hydrogen producing bioreactors is

being explored (Keskin et al. 2011; Oh et al. 2011; Stephen et al. 2017). The process by which PNS bacteria synthesizes hydrogen is called photo fermentation, this involves the stoichiometric conversion of a substrate to hydrogen and carbon dioxide. Nitrogenase enzyme catalyzes the hydrogen evolution with ATP serving as a co-factor. The enzyme is capable of reducing protons to hydrogen in the absence of other substrates. Appropriate substrates must possess a high C/N ratio as the nitrogenase is sensitive to the fixed nitrogen, but this is the case of many different wastes that might serve as feed stocks for photo fermentative production of hydrogen (Keskin et al. 2011). The onset of hydrogen photo production was delayed in the case of *Rhodobacter sphaeroides* O.U. 001 until the early stationary phase at C:N ratio of 15:2, in contrast to rapid hydrogen photo production by other PNS bacteria.(Sasikala et al. 1995). In general, the quantity and quality of light governs H_2 photoproduction in photosynthetic diazotrophs such as *R. palustris*. Considering this fact, modulation of photosynthesis using *N*-heterocyclic aromatic compounds, thereby escalating the hydrogen production was observed in *R. palustris*. A sum of 12- (671 µl H_2 mg dry wt^{-1} h^{-1}) and 6- (349 µl mg dry wt^{-1} h^{-1}) fold in H_2 photo production in *Rhodopseudomonas palustris* JA1 over 24 h was achieved with pyrazine 2-carboxylate (3 mM) and 3-picoline (3 mM), respectively (Archana et al. 2003). More recently, in an attempt to optimize the parameters including C:N ratio, P_0/V_L and I_0 the hydrogen production in *R. sphaeroides* DSM158, a stirred tank reactor was operated in continuous mode. It was observed that irradiation intensity had a greater effect on hydrogen production (165 mL L^{-1} h^{-1}) than volumetric power input from DSM158 (Krujatz et al. 2015). More studies of this type could open doors to wards BHP from PNS photosynthetic bacteria. In another study, hydrogen production from PNS bacteria was analyzed under varying salt concentrations and indicated less impact of salt concentration in producing photobiological H_2 (Adessi et al. 2016). Hence, the possibility of producing hydrogen on salt-containing substrates widens the range of feedstock that can be efficiently used in production processes. In addition, similar to the bio photolysis process, PNS strains with truncated photosynthetic antennas are likely to be obtaining higher efficiencies at high light intensities in photo fermentation as well.

Dark fermentation is another alternative to bio photolysis and photo fermentation to overcome long term research obligations like hydrogenase sensitivity to oxygen. Dark fermentation doesn't require direct capture of solar energy, and more importantly organic matter, such as plant and agricultural derived carbohydrates or industrial effluents, could be used as potential substrates. However, hydrogen yield per unit substrate is low for dark fermentation. *E. coli* strains have been observed to produce 2 mol of hydrogen per mol of glucose, as they use the pyruvate formate lyase (PFL) pathway. Metabolic engineering methodology has been used to enhance the yields in dark fermentation. Indeed, yields approaching this have been slightly augmented with suitably modified strains (Kim et al. 2011). Yet, only a 16.6% yield increase is represented, since 12 mol of hydrogen is theoretically available in glucose. In yet another interesting study, co-production of hydrogen and ethanol was studied in an *E. coli* Δ*pgi* mutant, with the over expression of PP pathway enzymes. Hydrogen at 1.69 mol and 1.50 mol ethanol from 1 mol glucose were produced, which was further improved to 1.74 mol and 1.62 mol, respectively with constitutive expression of the heterologous NADPH resistant genes (Sundara Sekar et al. 2017). Nonetheless, even this yield is unsuitable, as biofuels other than hydrogen could be extricated using current technologies from the same substrates at substantially higher yields, and still the excess carbon that is not converted to biofuels represents a serious waste treatment challenge. Also, E.coli has limited yield of 2 mol of hydrogen per mole of glucose, since it uses only pyruvate, formate lyase (PFL) pathway. In this regard, *Clostridium* sp. having the pyruvate ferredoxin oxidoreductase (PFOR) pathway are better targets for metabolic engineering since they have the potential capability of generating 4 mol of hydrogen per mole of glucose, which makes it 33% hydrogen yield per mol of glucose. In an attempt to understand the maximum H_2 production, *C. perfringens* strain JJC, *C. bifermentans* strain WYM and *Clostridium* sp. strain Ade.TY were studied in batch fermentation. The results showed the yields in the range of of 4.68 ± 0.12, 3.29 ± 0.11, and 2.87 ± 0.10 mol H_2/mol glucose, respectively for the above three strains (Wong et al. 2018). Hence, there is a huge scope for using Clostridium sp. for better yield of hydrogen, through dark fermentation. Although, until recently, many *Clostridia* sp. were relatively intractable genetically, yet with the recent development of better genetic tools, creating strains through rational engineering toward increased hydrogen production is now possible, and interesting results should be forthcoming (Mishra et al. 2019). In order to advance dark fermentation viablility it is essential that new and novel ways to extract more hydrogen energy be discerned.

Maximum substrate degradation to attain optimum hydrogen yields can be achieved by optimizing the operating conditions of the fermentation process. The above mentioned routes for biohydrogen production, including dark and photo-fermentation are less energy intensive (Hassan et al. 2018). Hence, this is a new approach to increase hydrogen yield from organic waste material and develop important and innovative energy recovery methods for hydrogen economy. Additionally, some studies (Chen et al. 2008; Mishra et al. 2016; Seifert et al. 2018) have observed that combining the use of dark fermentation with photo fermentation have proven to be successful in enhancing hydrogen production and further research in these combinations using different

chemical processes and feed stocks would be indispensable towards building a highly desirable hydrogen economy.

Use of microbial fuel cell technology as a carbon neutral process in BHP

Bio-chemical barriers that limit the bio hydrogen production to 33% could be overcome if an artificial pressure is applied to produce higher quantities of biological hydrogen. Microbial fuel cells (MFCs) are ideal machinery for escalating biological hydrogen production as reported previously (Cheng and Logan 2007; Zeng et al. 2015). To enhance further conversion of the residual organic acids, such as acetic acid, by bacteria, additional energy input using MFCs can be utilized. Electrogenesis is the process by which MFCs provide a direct method of obtaining bioelectricity from cellulose and other bio degradable organic matter. Electrogenic bacteria transfer electrons obtained through oxidation of organic matter outside the cell to the MFC anode while releasing protons into solution. Electrons, protons, and oxygen react at the cathode, generating water (Mahidhara et al. 2017; Mahidhara and Chintalapati 2015; Ramaprasad et al. 2018). Within MFCs, when oxidation of an electron donor at the anode with lower electrode potential (E_{ano}) is coupled to the reduction of an electron acceptor with a higher electrode potential (E_{cat}) at the cathode ($E_{cat} > E_{ano}$) a net positive cell voltage occurs (Rabaey and Rozendal 2010). Input of power is required when the oxidation of an electron donor at the anode is coupled to the reduction of an electron acceptor with a lower electrode potential at the cathode ($E_{cat} < E_{ano}$). For instance, when acetate/HCO_3 ($E' = -0.28$ V vs. SHE) is coupled to H^+/H_2 ($E' = -0.41$ V vs. SHE) the resulting cell voltage is negative (−0.13 V) requiring an external input of energy to drive the reaction. Based on thermodynamic analysis, the addition of 0.11 V to that generated by bacteria (0.13 V) should generate hydrogen gas at the cathode, but voltages of > 0.2 V are necessary because of electrode over potentials (Fig. 3). This hydrogen evolution process, referred to as electro hydrogenesis, provides a route for extending biohydrogen production past the endothermic barrier imposed by the microbial formation of fermentation dead-end products (Tremblay and Zhang 2015). MFCs linked after dark fermentation for BHP is a multistage process involving hydrogen fermentation, anaerobic digestion and MFC, in a sequential order. It was first demonstrated by simultaneous hydrogen and electricity production from a food processing wastewater by Sang Eun Oh and Logan (Oh and Logan 2005). Subsequent reports have innovatively addressed augmented biological production of hydrogen. To improve H_2 production rates, specially designed reactors based on MFCs were used with the addition of a small voltage to the circuit. The results showed

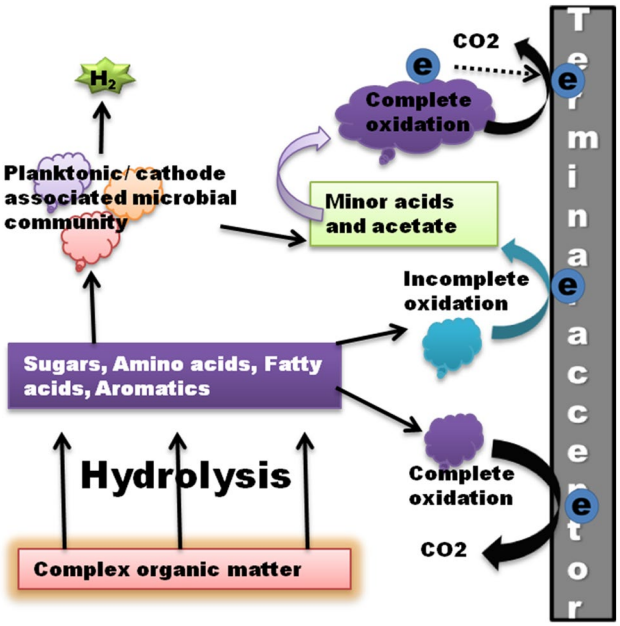

Fig. 3 Figure showing a broad variety of microbial metabolomics possibly occurs in a microbial electrolytic cell. Planktonic microbial community (For example: *Rhodopesudomonas*, *Pseudomonas*, *Paracoccus denitrificans*) degrades the hydrolysates of the complex organic matter in to short chain acids like acetate with the production of H_2, with a trivial exogenic electron transport. Electrogenic bacteria (*Pleobacter propionicus*, *Desulfotomaculum*, *Shewanella*, *Geobacter*) then can mediate complete degradation of these organic compounds. Bacterial communities such as *Paracoccus denitrificans* and *Geobacter* further mediate the oxidation of hydrogen with the help of hydrogenases

2.01–3.95 mol/mol hydrogen production at applied voltages of 0.2 to 0.8 V using acetic acid as a substrate (Cheng and Logan 2007). Maximum stoichiometric yields of 54–91% and overall energy efficiencies of 64–82%were reported in the same study using various carbon sources including acetic, butyric, lactic, propionic, and valeric acids, glucose and cellulose. Another report from the same laboratory showed salinity difference between river water and seawater can be captured in the form of H_2, with the use of microbial reverse electrolysis cells (MECs) having five alternative pairs of marine and river water cells, in the form of reverse electrodialysis (RED) cells (Kim and Logan 2011). These MECs have reportedly produced 0.29 to 0.59 m^3-H_2/m^3/day. In a more recent study, MECs were used to produce hydrogen, with the use of substrate without buffer solution, under continuous flow condition. Hydrogen gas was produced at a rate of 0.61 m^3-H_2/m^3/day, with a chemical oxygen demand (COD) removal efficiency of 81% and a coulombic efficiency of 41% (Song et al. 2016a). This study elucidates the possibility of using substrates without buffer solution in reverse MECs to generate hydrogen, which establishes an important step for

biological hydrogen production. Contemporarily, the anaerobic treatment of a high organic-strength wastewater-type feedstock was studied for energy recovery and organic matter removal using dark fermentation, anaerobic digestion and MFC technologies. MFCs were demonstrated to have high efficiency (92.4%) in removing the COD, where as hydrogen fermentation resulted in high specific energy yield (2277 J g^{-1} $COD_{removed}$ day^{-1}). In another study, a membrane less single chambered MEC was used to produce hydrogen by means of using carbon brush electrodes and decreasing the electrode spacing (Call and Logan 2008). Maximum hydrogen yield of 3.12 ± 0.02 m^3 H_2/m^3 reactor per day were reported which demonstrated the use of cost effective systems with simpler designs for BHP. Hence, electro-hydrogenesis from renewable and carbon–neutral biomass resources is much more efficient when used in combination with simplified MFC technology, through the addition of a small voltage to the circuit. More recently, MFCs coupled to microbial electrolytic cells were created to make the system self-sustained in terms of electric power. An MEC-MFC-coupled system for biohydrogen production from acetate was designed. Herein, hydrogen was produced in microbial electrolytic cell and the extra power was supplied by an MFC so that the system runs autonomously without external electric power supply (Sun et al. 2008). The cathodic hydrogen recovery was shown to be increased from 2.2 ± 0.2 to 14.9 ± 0.4 mL L^{-1} day^{-1}, with increase in phosphate buffer concentration. This setup provides an effective way for in situ utilization of the power generated from MFCs. In a similar study, self-powered submersible microbial electrolysis cells were designed to have more efficient BHP. A sum of 32.2 mL L^{-1} day^{-1} hydrogen was observed with 20 mM acetate buffer. Same microbial community structures in the anodic biofilms of the two cell units was also reported using 16S rRNA analysis, as a result of this novel operation (Zhang and Angelidaki 2012). In a more recent study, a MFC-MEC coupled system was established in order to enhance azo dye de-colorization, and the influence of several key factors on reactor performance was evaluated. The results showed that de-colorization rate in the coupled system had a 36.52–75.28% improvement compared to the single MFC, with anodic acetate concentration also exhibiting positive effects (Li et al. 2016). Four supercapacitive microbial fuel cells (SC-MFCs) with carbon brush anode and open-air cathode with free platinum and Pt group metal free catalysts (such as Fe-Aminoantipyrine (Fe-AAPyr) and Fe-Mebendazole (Fe-MBZ)), have been examined for hydrogen production, after being connected with an additional electrode. This electrode is connected with the anode of the first SC-MFC and placed in the fourth SC-MFC. The amount of hydrogen produced was 0.86 mM day^{-1} cm^{-2}(0.132 L day^{-1}) for Pt, 0.83 mM day^{-1} cm^{-2} (0.127 L day^{-1}) for Fe-AAPyr and 0.8 mM day^{-1} cm^{-2} (0.123 L day^{-1}) for Fe-MBZ. This setup showed simultaneous H_2 production and pulsed power generation with no need of external power sources (Santoro et al. 2016). The leftover organic matter (e.g. organic solid wastes, cane molasses, crude glycerol) obtained in a dark fermentation process are proposed to be the scratch for MFCs, to harness energy. For instance, cheese whey end product from dark fermentation was fed in MFCs and a constant current of 436 mv was reported, with dominant electrogenic communities like *Geobacter* and fermentative populations like *Lactobacillus*, in the bio anode (Wenzel et al. 2017). It is more interesting to see the development of such integrated systems linked to BHP production. In this view, the anode performance of a MEC was improved by combining it with an anaerobic reactor for high concentration industrial wastewater treatment. A pair of electrodes was inserted into an anaerobic reactor to form a MEC combined anaerobic digestion system, so that organics in wastewater can be degraded via anaerobic fermentation along with treatment of residual contamination using electrode reaction of MEC (Zhang et al. 2013). The results showed the addition of Fe^{+3} compounds along with an input of power of 0.8 V had reduced COD and enhanced dye reduction in the industrial effluents. Pyro sequencing also demonstrated the presence of more diverse microbial community including *Bacteroidetes, Chloroflexi, Proteobacteria* and *Firmicutes*; in the biofilm of the hybrid system, compared to common anaerobic reactor (Zhang et al. 2013). In another study, a dark fermentation reactor was connected with MFC to power MEC, to investigate hydrogen production from cellulose. Two MFCs connected in series to an MEC produced a maximum of 0.43 V using fermentation effluent as a feed, achieving a hydrogen production rate from the MEC of 0.48 m^3 H_2 m^{-3} day^{-1}. The overall hydrogen production for the integrated system (fermentation, MFC and MEC) was increased by 41% compared with fermentation alone to 14.3 mmol H(2)/g cellulose (Wang et al. 2011). More recently, a two-step protocol was standardized for efficient BHP from cheese whey. In the study, the effluents of anaerobic (methanogenic) digester were coupled to dark fermentative H_2-producing hydrogenogenic reactor utilizing the cheese whey and tested in the MEC process. Improved bio hydrogen production was reported from the effluent from methanogenic digester because of its lower carbohydrate- and higher volatile fatty acid contents. The authors advised methanogenesis as an advisable pretreatment step to enhance the H_2 formation from the acidic cheese whey (Rivera et al. 2017). These developments suggest that further design and development of dark fermentation -MFC technology in BHP is extremely worthwhile. Taken together, MXCs (either MFC or MEC), can be successfully

operated with BHP systems such as dark fermentation, to achieve absolute carbon neutral processes. This further depends on the requirements such as (i) available feed stock and/or substrate utilization efficiency by the bacteria (ii) favored end product such as hydrogen or electricity and (iii) scale up costs to produce sustainable products.

Storage and transport of hydrogen

Around 3 kg of hydrogen is lost to the atmosphere per second, why this happens is not yet fully understood (Zahnle and Catling 2009). A powerful process is required in order to best utilize the hydrogen present on earth. Although molecular hydrogen has very high energy density on a mass basis, partly because of its low molecular weight, as a gas at ambient conditions it has very low energy density by volume. For instance, a vehicle that uses hydrogen requires a 3000 times bigger tank capacity compared to a vehicle that uses gasoline. Hence, based on this property, it is extremely challenging to store and transport hydrogen. If it is to be used as fuel stored on board in a vehicle, pure hydrogen gas must be stored in an energy-dense form to provide satisfactory driving range. To deal with high gravimetric energy density of the hydrogen compared to its low volumetric energy density, multiple investigations have been conducted, exclusively in the storage and transportation sector.

Based on type IV carbon-composite technology (Delucchi et al. 2014), compressed hydrogen in hydrogen tanks at 350 bar (5000 psi) and 700 bar (10,000 psi) is used for hydrogen tank systems in vehicles. Hydrogen can also be liquefied by reducing its temperature to − 253 °C, which significantly reduces its storage volume (Sadaghiani and Mehrpooya 2017). As solids have less entropy, compared to gaseous and liquid states, storage of hydrogen in solid metal/ non metal hydride form was also investigated (Kwon et al. 2019). Chemical reactions such as hydrolysis reactions, hydrogenation/dehydrogenation reactions are employed to achieve these objectives. Weakly bound metal hydrides with transition metals and complex metal hydrides, including the hydrides of magnesium, sodium, lithium, or calcium and aluminum or boron are found to have significant advantages for hydrogen economy (Seenithurai and Chai 2018). Another highly significant option in storage of hydrogen for the future is the utilization of nanotechnology (Schneemann and White 2018). Semiconductor nano-materials, such asTiO_2 and cadmium sulfide nanostructures, have been studied as efficient catalysts for water conversion into oxygen and hydrogen (Ma et al. 2019; Vinokurov et al. 2017). Nano-structured carbons, metal–organic frameworks and polymers are greatly investigated for their applications in hydrogen storage and transportation (Wood et al. 2017). Hence, practices such as onsite generation; storing as compressed gas; using pipelines to transport; using as a liquid fuel; storage as a solid metal hydrides are the ideal methods for storage and transportation of hydrogen.

Utilization of hydrogen energy

The most effective way to convert hydrogen and oxygen into water is using a fuel cell. As this process is a highly exergonic reaction, energy is released, which is captured as electrical energy. Thus, chemical energy is converted into electrical energy. Ultimately, a fuel cell enables hydrogen and oxygen to blend in an electrochemical reaction (Fig. 1b). The result is a spontaneous reaction, which results in the production of electricity, water, and heat. Both the fuel cells and batteries resemble each other in the sense that they both generate useful electric power from the energy generated in the electrochemical reaction. Moreover, generation of electric power by the fuel cell is proceeded, until the complete utilization of its hydrogen fuel (Ebrahimzadeh et al. 2018). Fuel cells represent a potential technology for use as a source of electricity and heat for buildings. Additionally, power for electric and hybrid vehicles can also be made using fuel cells. Although, pure hydrogen is a source of energy, other fuels such as gasoline, methanol, or natural gas can be reformed to generate the needed hydrogen for fuel cells. As previously mentioned, metal and non-metallic hydrides along with nano-materials are also acknowledged as redeemable substances. Taken together, with the fast-moving technology, hydrogen could come on par with electricity as a vital energy carrier. Some renewable energy sources such as wind and sun may not be able to generate energy around the clock, yet are able to produce hydrogen and electric power potentially stored for later use. Thus, complementing the drawbacks of one renewable technology with others will help in the generation of a sustainable energy infrastructure.

Summary

BHP is a challenging area of biotechnology in establishing hydrogen as a source of energy capable of high, renewable yields. Combination of ionic species such as hydrogen (H^+) and electrons (e^-) into hydrogen gas (H_2) is endergonic, which makes it an expansive process. Ultimately, utilizing special enzymes called hydrogenases and nitrogenases, separately or combined with dark fermentation process can catalyze the formation of hydrogen in biological systems, which are fundamentally more economical than the current use of fossil fuels. Drawbacks within biological systems, such as oxygen sensitivity of enzymes and stoichiometric yields below 33% in the case of dark fermentation, could potentially, by means of molecular biology and metabolic engineering technologies, be effectively overcome. Further research into effectively utilizing wastewater and

biomass-based materials as a crucial feedstock element in BHP would also be beneficial towards greatly improving other environmental aspects, whilst also producing renewable energy for consumption without the necessity of fossil fuels (Wang and Yin 2018). In addition, advances in microbial fuel cell technologies and electrolytic cells or a combination of both are promising to be highly beneficial towards developing biological hydrogen economy.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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